iGEM Protein Cages Team

Lab Journal

June 13th PC Journal

Goals for today: We have a vector of O333 from Neil King contained in a pET vector, the goal for today is to transform this vector, grow it tomorrow and PCR it for a large number of copies to work with and creation of a biobrick. Primers will need to be designed including the T7 promoter to terminator, with iGEM prefix and suffix.

Made plates

- 12.5g LB
- 7.5 Bacto Agar
- 500mL dH2O
- 500uL Kanamycin (50mg/mL)

- 20 minutes autoclave
- 4 hours total time to solidify
  => 32 plates total

Transformation

- 30uL iGEM electrocompetent cells
- 1uL O333 vector 1:100 dilution
- 970uL SOC save

- 45 minutes incubation @ 800rpm/37C
- 1 and 1:10 dilution plating
  => 3 transformations total
  => arc time: 5.5s, 5.6s, 5.6s

**Plates in 37C inoculation from 6:40pm

Conclusions: Transformations had a consistent arc time. Tomorrow morning plates will be checked for colonies and grown.

June 14th Lab Journal

Recap from previous day: No colonies were shown from any of the transformations. Competent cells may have been of low quality (relatively long arc times).

Goals: Today, commercial electro-competent cells are going to be transformed, and different concentrations of DNA will be tested for transformation as well.

Transformation:

- 3x 25uL commercial electrocompetent cells
- 1uL of 1:5, 1:50, 1:100 serial dilutions of plasmid from Neil King
- 970uL SOC save

- 60 minutes save (800rpm @ 37C), then additional 50 minutes at 37C incubator (no shaking) as I was waiting for plating beads to be autoclaved and cooled
- 1 and 1:10 dilution platings
  => 1:5 DNA: 5.1s arc time
  => 1:50 DNA: 5.0s arc time
  => 1:100 DNA: 4.8s arc time

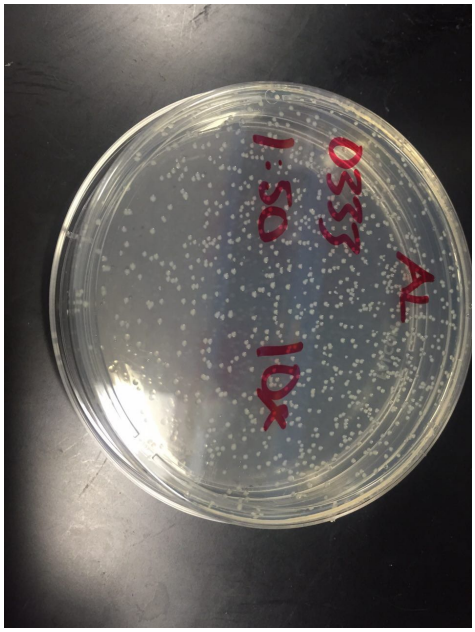
**Plates in 37C incubation from 4:10pm

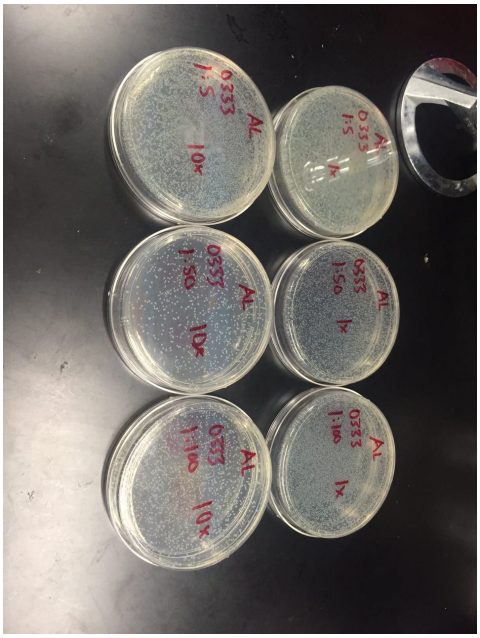
Conclusions: Same as yesterday, though today **arc times were slightly better.** Unfortunately, my timing was off for plating because I was waiting to autoclave new beads (thanks for sharing, grad students!), so when I plated the transformants smelled pretty awful. Hopefully that doesn't make too much of a difference.

June 15th Lab Journal

Recap from previous day: Colonies on all transformations were successful. Interestingly, the 1:50 dilution of DNA produced the largest number of colonies. All plates were very full, with the 1x plating dilutions being entirely unusable due to how covered they were. This may also be due to the long saving time from yesterday.

I decided to pick colonies from the 10x plating dilution of the 1:50 transformation.





Goals: The only thing I'll really be able to do is pick colonies and grow them overnight for tomorrow.

Inoculation:

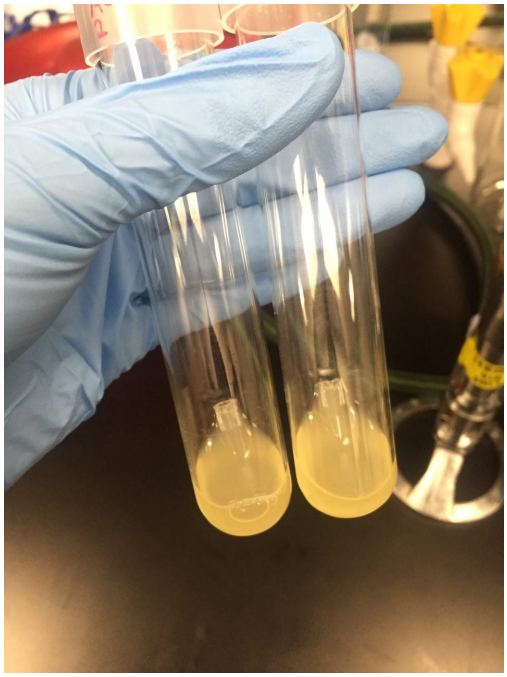
- Autoclaved culture tubes
- 2 isolated colonies picked
- 5mL LB per culture tube

**Tubes in 37C incubator from 3:30pm

Conclusions: The commercial cells were far more successful than our old cells. I used two colonies, which may not end up perfect, so I've stored the plate in a 4C cooler. Tomorrow, I'll store the cells and/or miniprep and possibly make glycerol stocks of them, though I don't have primers to sequence or PCR them beyond that.

June 16th Lab Journal

Recap from previous day: Both stab cultures grew nicely.



Goals for today: I have to leave early today, so I'll be moving the cultures into 4C fridge until tomorrow when I have primers ready.

Ordered primers

- T7 (sequencing)
- T7 Term (sequencing)
- NK O3-33 Forward + prefix
- NK O3-33 Reverse + suffix

Conclusions: Nothing special today. Tomorrow when the primers arrive, I will make glycerol stocks, miniprep, sequence and possibly pcr.

June 17th Lab Journal

Recap from previous day: Ordered primers for PCR and sequencing yesterday.

Goals for today: The stab cultures should be divided up, mostly for miniprepping and a bit to make glycerol stocks. If the primers arrive, I'll run a PCR, but if not, I'll just try to complete miniprep and make stocks and dump the remaining culture.

Miniprep

- 3mL of each culture (4 microcentrifuge tubes running simultaneously)
- 200uL P1 buffer
- 200uL P2 buffer
- 400uL P3 buffer (stored in 4C)
- 40uL DNA binding buffer
- *All buffer volumes are per tube*

- 16000g for all centrifuges
- 2 minutes for pellet
- Vortex with P1 to resuspend
- 1:40 P2 buffer 6 inversions
- P3 buffer 8 inversions
- (remaining steps just followed protocol @ 16000g)
- 2 tubes of each culture miniprepped, mixed them at the end
  => Culture 1: 40.01ng/uL, 260/280: 1.71
  => Culture 2: 39.07ng/uL, 260/280: 1.74

June 21 Lab Journal

Goals: Miniprep pcquad and O333 to isolate plasmid for transformation. Also need to send O333 for sequencing.

Miniprep

- pcquad 3.0 1 cells from -80 freezer
- O333 cells from agar plate made by Andy.
- Chloramphenicol and Kanamycin from freezer
- 10 mL LB

- 5mL LB into 2 culture tubes
- 5uL Chloramphenicol into one, 5uL Kan into other
- Stab culture into culture tubes, O333 with Kan
- Shaker at 3:30 PM

=>2 5mL cultures for miniprep

=>Only O333 worked, sent for seuquencing

Conclusion: To be taken out at 10 AM, caution for long growing time (18hrs)

June 22 Lab Journal

Recap from previous day: O333 got plasmid, sent for sequencing today, pcquad grew too much and had to be redone.

Goals: Redo pcquad miniprep.

Miniprep

- pcquad 3.0 1 cells from -80 freezer
- Chloramphenicol from freezer
- 5 mL LB

- 5mL LB into 1 culture tube
- 5uL Chloramphenicol added
- Stab culture into culture tube
- Shaker at 5:30 PM

=>5mL culture for miniprep

Conclusions: Similar as yesterday, but placed in shaker later so hopefully better result.

June 23 Lab Journal

Goals: Design Pcr to create O3-33 mutants

Mutant Generation

- Decided to just synthesize O3-33 mutants

Conclusions: None

June 24 Lab Journal

Recap: Sequence has been confirmed for O333 and both it and pcquad 3.0 are in plasmid form.

Goals: Grow and miniprep more mutants of pcquad previously created by Phillip.

Growth

- pcquad cells from -80 freezer
    - specifically mutants 2, 13, 4.3, and 4.4
- Chloramphenicol from freezer
- 20 mL LB

- 5mL LB into 4 culture tubes each
- 5uL Chloramphenicol added to each
- Stab culture into culture tubes
- Shaker at 5:30 PM

=>4 5mL cultures for miniprep

Designed ferritin mutants

Conclusions: Ready to miniprep by the 25th, done by Yashes

June 25 Lab Journal

Goals: Miniprep pcQaud mutants

Miniprep

- M4-4: Concentration: 238.37 ng/uL, 260/280: 1.82
- M2: Concentration: 305.00 ng/uL, 260/280: 1.82
- M13: Concentration: 258.71 ng/uL, 260/280: 1.79
- M4-3: Concentration: 252.41 ng/uL, 260/280: 1.82

Conclusions: None

June 26 Lab Journal

Recap: Yashes completed miniprep, with an end result of 4 plasmids. This is in addition to the pc 3.0 and O333 from earlier

Goals: Transform all 6 plasmids chemically into new BL21 cells and plate.

Transformation

- 30uL BL21 cells
- approx 5 ng plasmid
- 970 uL SOC
- 10 Cat plates
- 2 Kan plates for O333

● Combined plasmid, cells, and SOC, put in shaker at 37C for 1 hr, 6 tubes total
● Spun down at 6000rpm for 4 mins, poured off old SOC
● Resuspended in 110uL SOC, diluted 1 to 100 with SOC
    ○ =>12 total tubes of cells in SOC.
● Plated 100uL on plates, spread with beads and placed in the incubator at 37C overnight

=>cultures for pcquad 3.0 and mutants 2, 13, 4.3, 4.4 and O333

Update: Transformations all worked. 1:1 dilutions worked the best, giving us most colonies.

Conclusions: Colonies grew successfully and were used to create starter cultures for expression

June 27 Lab Journal

Goals: Innoculate PCQuad 3.0 and O3-33 in small culture(5 mL), Design Ferritin Mutants, contact Protein Expression Core to gain access to sonicator, order Mutants if email comes back, Order Primers(to clone O3-33 into biobrick) if email come back, Order Thrombin

Innoculated 3 of PCQuad 3.0 and 1 of O3-33 in small culture(5 mL)

Designed Ferritin Mutants

Contacted and gained access to sonicator

Thrombin

● Bovine Thrombin:
    ○ 1-2 ug per batch of protein
    ○ Need 10 units for every 5 ug of protein so 2 units/ug

- 4-6 units of thrombin per batch of protein

Conclusions: None

June 28 Lab Journal

Goals: Grow up large cultures of O3-33 and PCquad 3.0 and induce protein expression, read up on O3-33 Purification

Grew up large cultures of O3-33 and PCquad 3.0 and induced protein expression

- Transferred all 5mL of starter culture into 350 mL culture(3 for PCquad and 1 for O3-33)
- Grew O3-33 and 1 PCquad using O3-33 protocol
  - Grow to OD of **.8 in 37C**
  - Induce protein expression at **30C for 5 hours** at .5mM of IPTG

Time: **Start: 12:03**	OD(PC)	OD(O3-33)
2:10	.155	.105
3:10	.576	.574
3:30	.778	.817
3:45	**Induced using .0425 g IPTG**	**Induced using .0425 g IPTG**

- Grew 2 PCquads using PCQuad protocol
  - Grow to OD of **.6 in 37 C**
  - Induce protein expression at **37 C for 4 hours** at .5mM of IPTG

Time: **Start: 1:27**	OD(PC 2)	OD (PC 3)
2:50	.043	.054
3:50	.094	.152
4:50	.184	.417
-		.638
5:43	.512	**Induced using .0425 g IPTG**

6:45	**Induced using .0425 g IPTG**	

- After Induction time was complete, cultures were transferred to falcon tubes and spun down(10 mins)
- Pellet washed with cold water and spun down again
- Pellet stored in -80 C

Conclusions: Need to do colony PCR to verify if plasmid in things we grew. Took a small amount of pellet from each sample and stored.

June 29 Lab Journal

Goals: Run PCR for O3-33 and PCquads and run pcrs from colonies, Make Purification Buffers

Ran PCR

- 2 colonies of PCquad 3.0 and 2 colonies of 03-33(from plates)
- 1 colony from eac mutant(from plate)
- 1 colony of each culture grown(O3-33, PCquad 3.0, PCquad 3.0 (2), PCquad 3.0 (3)
    - Did 1:1, 1:10, 1:100 dilutions

Made Buffers.

- (Missing DTT for one of them) → Check buffers under project for details on concentration
- Created buffers for O3-33 and Pcquad purification
- O3-33:

- Lysis buffer: 50 mM TRIS (ph=8.0), 250 mM NaCl, 20mM imidazole supplemented with 1 mM phenylmethanesulfonyl fluoride
    - 50 ml solution
        - 2.5 mL TRIS
        - 0.7305 g NaCl
        - 0.068 g imidazole
        - 0.0087 g phenylmethanesulfonyl fluoride
        - Up to 50 ml water
- Running buffer: 25 mM TRIS pH 8.0, 150 mM NaCl, 1 mM DTT
    - 50 ml solution
        - 1.25 mL TRIS
        - 0.438 g NaCl
        - 0.00771 g DTT
        - Up to 50 mL water

- PCQuad:
    - Lysis buffer: 20mM sodium phosphate (ph=8.0), 300 mM sodium chloride, 10 mM imidazole
        - 50 ml solution
            - .164 g sodium phosphate
            - .877 g NaCl
            - .034 g imidazole
            - Up to 50 ml water
    - Lysis buffer supplemented with 500 mM imidazole
        - 50 ml solution
            - 1.702 g imidazole
            - Up to 50 ml lysis buffer
    - Dialysis buffer: 20mM TRIS buffer (ph = 8.0), 100 mM sodium chloride, 2mM beta-mercaptoethanol
        - 50 ml solution
            - 1 ml TRIS
            - 0.2922 g NaCl
            - 0.007 ml beta-mercaptoethanol
            - Up to 50 ml water
    - Running buffer: same as dialysis buffer

    - Buffers finally created:
        - 100 ml of O3-33 lysis buffer
        - About 100 ml of pcquad lysis buffer
        - 50 ml pcquad lysis buffer supplemented with 500 mM imidazole
        - 200 ml of pcquad dialysis buffer
        - Could not finish 200 ml of O3-33 running buffer because did not have DTT
            - Need to order DTT or borrow from another lab

Conclusions: Only 03-33 Cages showed up. Might have messed up with PCquad primers. Redo PCquad pcr tmro.

June 30 Lab Journal

Goals: Write Abstract, Run PCR of PCQuad with right primers, Order DTT, Order Thrombin, Iron out Protein Purification Protocol, Send out follow-up email, Redo Transformation just in case, Order Mutants, Order Mutant Amp Primers, Order "extraction" primers for O3-33

Retransformation of pcquad failed

Colony PCR

- Realized used wrong primers for PCR of pcquad from colony, pcquad and pellet
- Rerunning pcr on pcquad (total 5)
    - 1 from each of 3 pcquad pellets (total 3)
    - 1 colony from plate
    - 1 from original pcquad miniprepped plasmid
    - All showed except pcquad 3.0 (3)

Buffer Preparation

- Finished running buffer for O3-33 purification
    - Using BME in replace of DTT

Conclusions: None

July 1 Lab Journal

Goals: Purify proteins

Purification

- Purified O3-33, Pcquad 3.0 (1), and Pcquad 3.0 (2)
    - Suspended pellets in lysis buffer
    - Lysed cells by sonification in appropriate lysis buffer
        - Lysed O3-33 for 5 min continuous (may have degraded protein oops)
        - Lysed Pcquad 3.0 (1) and pcquad 3.0 (2) in 10 second bursts
    - Filtered out lysate using millipore filtration tubes
        - Ended up with:
            - 10 ml of pcquad 3.0 (2)
            - 7.5 ml of O3-33

            - 5 ml of Pcquad 3.0 (1) (difficult to filter, very cloudy, may be because O3-33 protocol was followed (i.e. inoculated for 5 instead of 4 hours)
        - Added filtered protein to equilibration buffer (20 mM imidazole)
        - Prepared purification columns by adding about 1 ml of resin
        - Dripped protein in eq. buffer through column twice
        - Washed protein 3 times in:
            - O3-33: 50 mM imidazole
            - Pcquad: 25 mM imidazole
        - Eluted protein 3 times in:
            - 200 mM, 350 mM and 500 mM imidazole

Conclusions: Have a general purification protocol, but may need to change buffer concentrations

July 5 Lab Journal

Goals: Run SDS Page Gel

Buffer Preparation

- Made 400 ml stock of O3-33 lysis buffer and Pcquad lysis buffer
    - Also found DTT in lab

SDS Page

- Ran SDS Page on purified protein (12 wells with desired protein plus 1 ladder):
    - Wash 3 + 3 Elution steps for O3-33, pcquad 3.0 (1), and pcquad 3.0 (2)
- Stained and destained gel

Conclusions: Will see Gel Results

July 6 Lab Journal

Goals: Colony PCR

Colony PCR

- Ran colony pcr on three new O3-33 colonies and three Pcquad 3.0 colonies

Conclusions; None

July 7-10 Lab Journal

Goals: Innnoculate and grow out bacteria

Inoculating/growing

- 2 O3-33, 2 Pc, 2 Controls
    - Why did this take so long: Failed innoculation multiple times

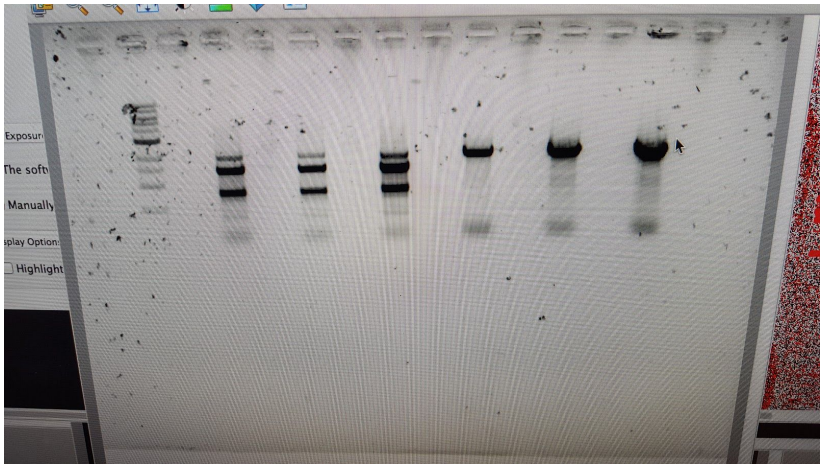
Colony PCR

Ran colony Pcr on three O3-33 and three PcQuad colonies with the following protol:

ddH20	7.5 ul
APEX Taq	12.5 ul
Primer F	2.0 ul
Primer R	2.0 ul
Template	1.0 ul

25 ul reactions
Cycle:
95。5 min
25x cycles: 95。30s, 42。(o3-33)/ 47。(pcQuad) 45s, 72。42s (o3-33) 72s (pcQuad)
72。3 min
10。infinite

From left to right: Ladder, O3-33:1,2,3; PcQuad: 1,2,3



Lots of background due to wrong cycling, but band at correct length appears in all samples.

Conclusion: Forshadowing out cloning problems to come

July 11 Lab Journal

Goals: Purify protein from bacteria

Purification

- Lysed 2 O3-33 colonies, 2 Pcquad colonies and 2 controls (1 pc, 1 O3)
    - O31: forgot to chill in ice for ten minutes after adding lysis buffer before sonicating
    - PMSF was not completely dissolved so may not have been transferred properly
    - PC2: chilled in lysis buffer for longer than 10 minutes
- Centrifuged lysates down for 45 minutes at 13,000 rpm
- Filtered supernatant to get protein
- Protein purification (2 methods: column and batch)
    - Pc + C1: Wash - 25,25,50, Elution - 150,250,400
    - O3 + C2: Wash - 30,40,50, Elution - 150,250,400

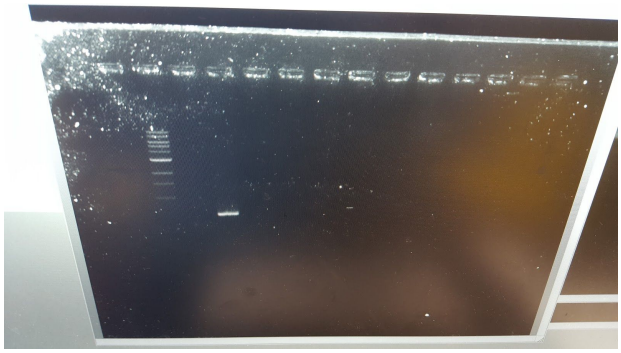
O3-33 Cloning

Ran a PCR with O3-33 amplification primers Forward 1 and Reverse 1 to begin the process of extracting the O3-33 gene without the illegal iGem site into PSB123 for a biobrick.

Protocol:

Q5 2x Master Mix	25 ul
Water	19 ul
F1 primer 10 uM	2.5 ul
R1 primer 10 uM	2.5 ul
Template (1:50)	1 ul

Results:



Initially incorrectly concluded this was primer dimer when in reality the DNA ladder at 0.5kbp did not show up

Conclusions: Will run a gel on purification products, will continue cloning

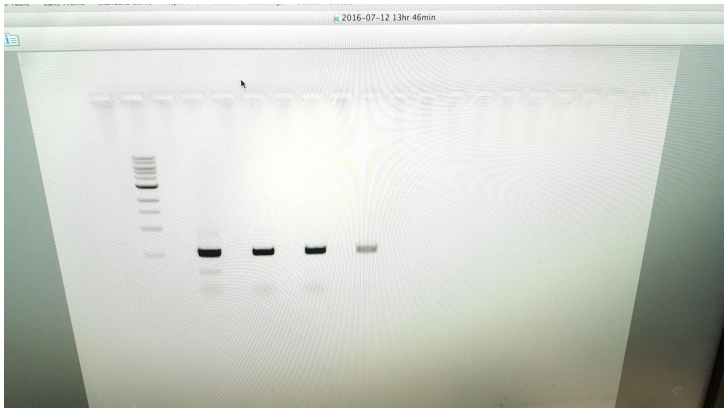
July 12 Lab Journal

Goals: Run a gel on protein products from yesterday, continue cloning

SDS Page

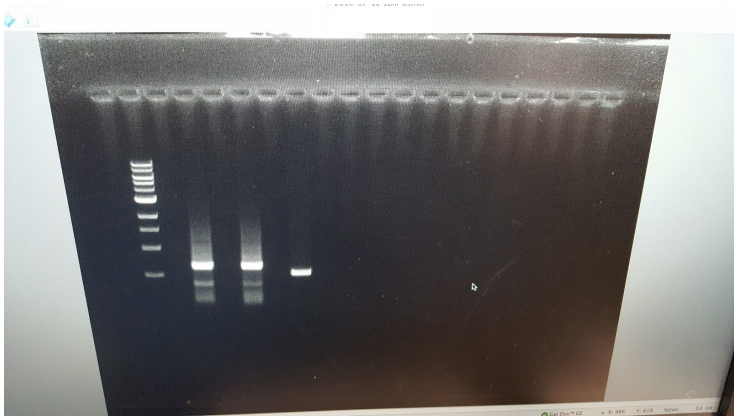
- Run SDS-Page on purified proteins from yesterday
- Stained and destained

O3-33 Cloning



Re ran yesterday's PCR with the same conditions. From L to R: ladder, miniprep plasmid, 1:50 dilution, 1:100 dilution, yesterday's pcr product

This time the .5kbp band showed up, so proceeded on with the second PCR using primers F2 and R2. Unfortunately forgot to purify before, so purified yesterday's PCR product: 98 ng/ul extracted (10ul)
Ran PCR again with the purified product:



From L to R: Ladder, Purified PCR-2 product, Raw PCR-2 product, PCR-1 product

Since PCR 2 product(s) are larger in length, we can confirm that the primers worked.

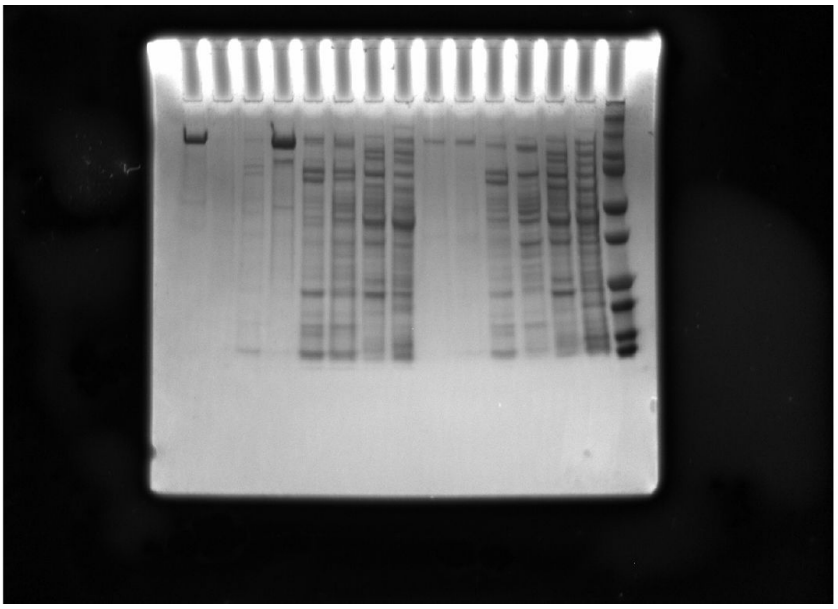
Conclusions: Will image gel tomorrow, continue cloning

July 13 Lab Journal

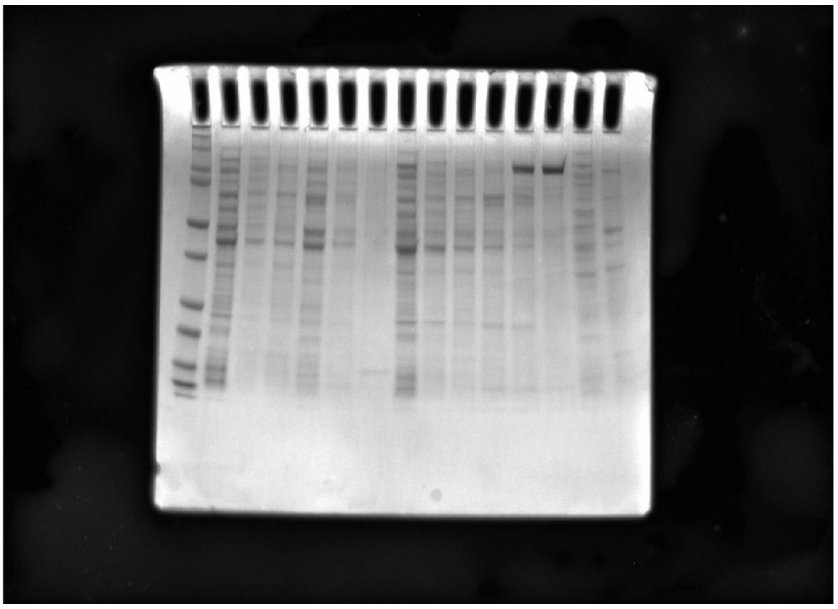
Goals: Image Gels

SDS Page Gels

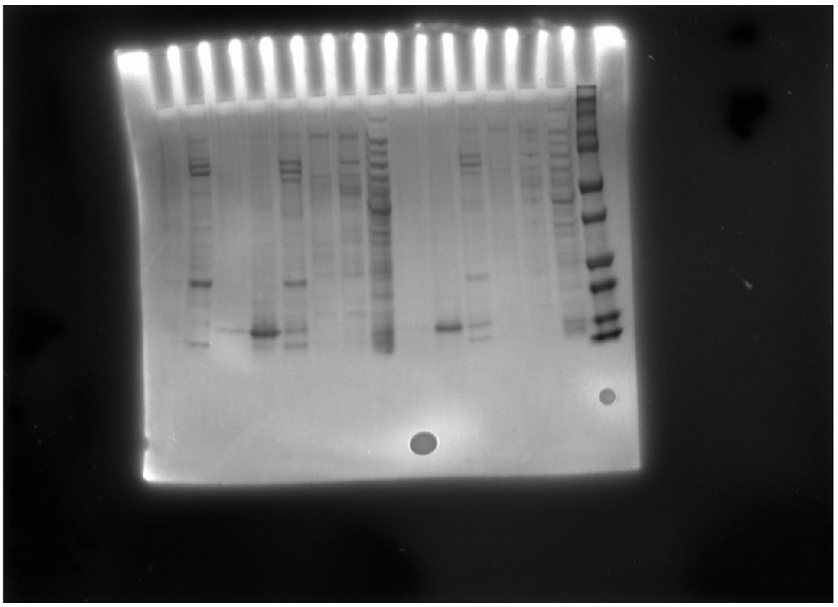
- SDS-Page from purified proteins:
- Column purification PC: Order: ladder, Pc 1: w1, w2, w3, e1, e2, e3, PC 2: w1, w2, w3, e1, e2, C1: e2, e3, Pc 2: e3



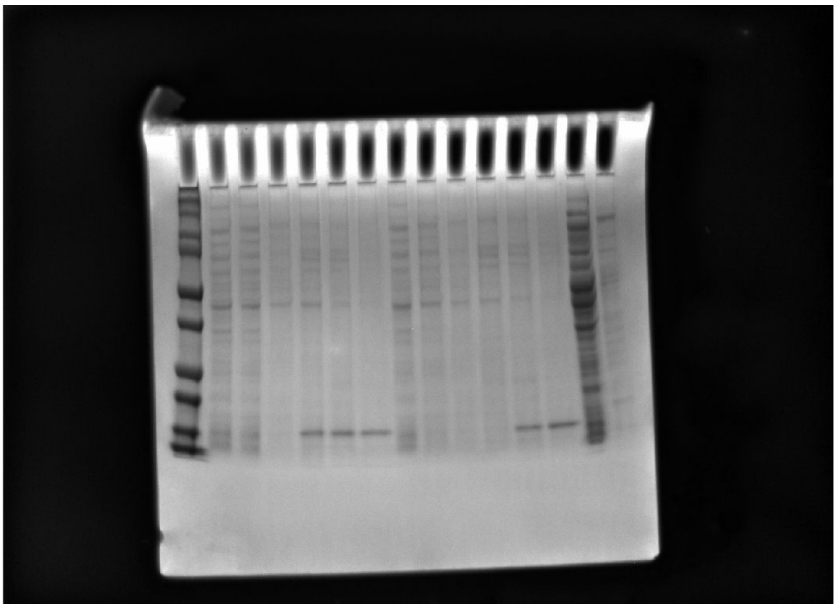
- Batch purification PC: Order: ladder, Pc 1: w1, w2, w3, e1, e2, e3, PC 2: w1, w2, w3, e1, e2, e3, C1: w1, w3



- Column purification O3: Order: ladder, O3 1: w1, w2, w3, e1, e2, e3, O3 2: w1, w2, w3, e1, e2, e3, C2 : e2, e3



- Batch purification O3: Order: ladder, O3 1: w1, w2, w3, e1, e2, e3, O3 2: w1, w2, w3, e1, e2, e3, C2: w1, w3



Things to order:

- Native page gel sample buffer Biorad
- PAGE gels from GenScript
- Imidizole
- MES (for resin regeneration)

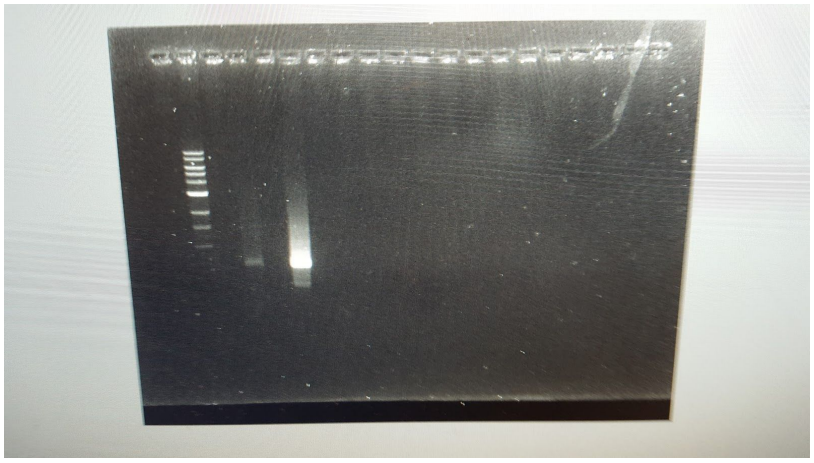
Conclusions: Have some protein expression. Need to run a DLS on the samples now

July 14 Lab Journal

Goals: Continue cloning

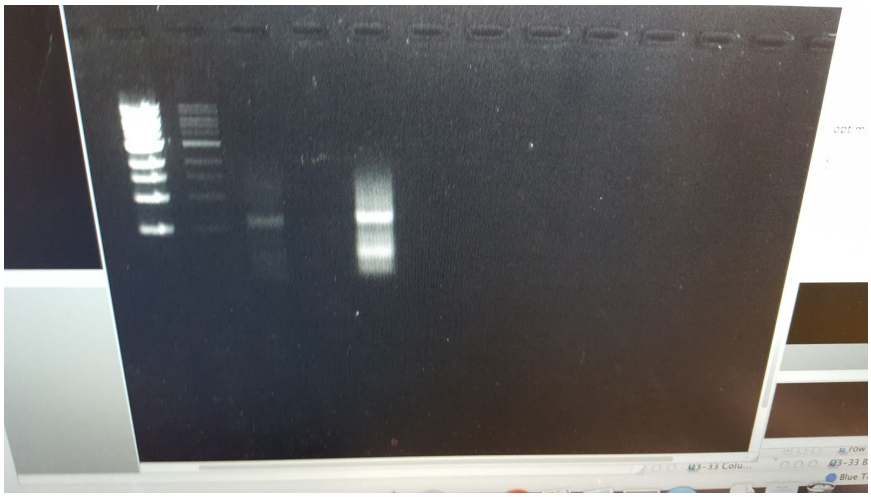
Cloning

Ran 2 pcrs, one with F3 and R3 primers and one with F4 and R4 primers. The first product was pretty low:



From left to right: Ladder, PCR #3 (one of interest), PCR #2

So I purified the DNA, yielding only about 60 ng/ul and ran the next PCR:



From left to right: Ladder, Ladder spillout, PCR#3, PCR#4 (of interest)

Although it's not pretty, the band is at the right size (at a larger length). All the noise will be removed by gel extraction tomorrow.

Conclusions: Continue cloning

July 16 Lab Journal

Goals: Continue Cloning

Digestion

PCR purified PCR #4's product and ran a digestion according to following protocol:

DNA	7.7ul
10X NEB 2.1	5ul
EcoRI	0.5ul
PstI	0;.5ul
H2O	36.3ul

@ 37C 1 hr, 80C 20min

Then ran a gel.
Strong band showed up at correct size.
And then extracted the DNA from the gel.Will continue my ligation on Monday.

Conclusions: Continue Cloning

July 19 Lab Journal

Goals: Clone pcquad mutants from gene block because old mutants were not valid (invalid sequence), Regenerate resin so we can start doing purification

Cloning (Digestion, ligation, transformation)

Realized that did not digest plasmid, so digest the plasmid using nearly the same protocol as before for the pcr product. After digestion, ligated according to following protocol:

10x T4 Buffer	2ul
pSB123 Vector	1.44ul
Insert	4.18ul
H2O	11.38ul
T4 DNA Ligase	1ul

In a 20ul reaction. Following the addgene protocol:

- Gently mix the reaction by pipetting up and down and microfuge briefly.
- For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
- Heat inactivate at 65°C for 10 minutes. (accidentally did it for 15 min)
- Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells. (added 5ul)

Transformed the BL21 cells according to following protocol:

1. Take competent cells out of -80°C and thaw on ice (approximately 20-30min).
2. Take agar plates (containing the appropriate antibiotic) out of 4°C to warm up to room temperature or place in 37°C incubator.
3. Mix 1 to 5µl of DNA (usually 10pg to 100ng) into 20-50µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times. (I added 5ul)
4. Place the competent cell/DNA mixture on ice for 20-30min.
5. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 seconds (45sec is usually ideal, but this varies depending on the competent cells you are using).
6. Put the tubes back on ice for 2 min.
7. Add 250-500µl LB or SOC media (without antibiotic) and grow in 37°C shaking incubator for 45min.
8. Plate some or all of the transformation onto a 10cm LB agar plate containing the appropriate antibiotic.
9. Incubate plates at 37°C overnight.

***** Couple of modifications necessary: Another website said that BL21 cells need to be heat shocked for only 10 seconds, and I did them for about 45 seconds, so I may end up not getting any product********

Will see tomorrow if I get any growth (did 1:1 and 1:10 cat agar plates)

Conclusions: Used the wrong transformation protocol; need to use the zymo one. Also ligation should be 1hr, with 20 min at 65 for deactivation of ligase

July 22 Lab Journal

Goals: DLS, Cloning

Dynamic Light Scattering

- Ran DLS on O3-33 2 and PC 2 column and batch purifications

Cloning

- Continued cloning Pcquad mutants, O3-33 mutants, and O3-33 wild type

Native Gel

- Ran native gel on O3-33 and Pc

Conclusions: DLS shows promising results, but native gel did not work for some reason

July 23 Lab Journal

Goals: Miniprep, send mutant 2 for sequencing

Miniprep

- Miniprepped Pcquad mutant 2 (4 batches)
    - 1: 384.29 ng/uL
    - 2: 426.88 ng/uL
    - 3: 548.42 ng/uL
    - 4: 561.67 ng/uL

Sequencing

- Need to send mutant 2 for sequencing
- UPDATE: Sequence was good at blocks 2 and 3, but not at the t7 promotor
    - Suspect we cut out T7 promotor in out PSC1C3
    - The T7 promotor and RBS are suspected to be between prefix and suffix

Conclusions: Need to redo PCR for all cloning

July 27 Lab Journal

Goals: Continue pcr series to clone mutants of O3 into biobrick

PCR

- Q5 Master Mix 2X
- Water
- Primers with overhang
- 3 mutants from previous pcr
- Gel, ladder, and loading dye

Cloning

- Created 25uL reaction for mutants, mutants 1 and 3 on step 3, mutant 2 on step 4 of 4.
- Ran PCR, extension time about 20 sec, annealing 64C for step 3, 57 for step 4
- Ran on gel, only mutant 1 successfully amplified
  =>Mutant 1 ready for last pcr step

Conclusion: Still having some difficulties with reactions. However, it was discovered that the plasmid to be used actually had the promoter and RBS desired, so new primers were ordered for one step of pcr that would allow easier cloning.

July 28 Lab Journal

Goals: Purify pcquad proteins (PC 13, 2), continue with cloning, run a SDS Page Gel

MOPS Buffer with SDS

- Made using GenScript's protocol from the gel manual

Protein Purification

- Purified PC 13 and 2 using nickel columns
- Ran a gel on washes and elutions for these
- Stained gel with Coomassie Blue
- Destained gel with destaining solution
- Made a container for coomassie blue and destaining solution waste

Cloning

- Discovered major flaw in the primer Reverse 2 for O3-33 wt
    - Ordered new primers accordingly, so that only one pcr will be needed, as Spe and Xba sites will be used
- PCR yields were still low

Conclusions: Run PCR with more cycles, cannot use the Tris-glycine running buffer for native gels, all the PCR Gunvant has been doing is trash THANKS SCOTT

July 29 Lab Journal

Goals: Image gel, continue cloning

SDS Page Gel

- Destaining solution evaporated
- Gel did not show the protein, probably because the sequence was incorrect as previously determined

Cloning

- Got decent yield for all of the pcQuad mutants, so can move on with digestion.

****Summary of Current Cloning Plans****

- Scott

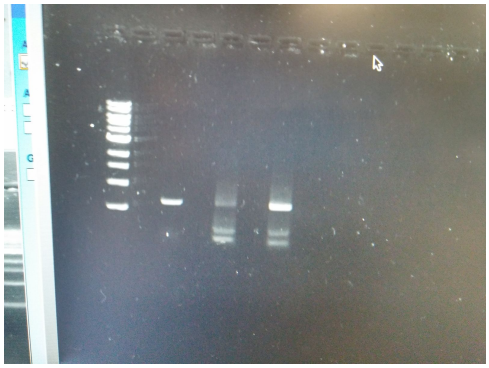
    - Cloning O3-33 mutants using mutant reverse with the new PCT-F1 primer
    - Cutting at Xba & PstI
    - **To be Confirmed**
- Nithin/Yashes
    - Cloning pcQuad mutants using old primers from Philip
    - Cutting at Xba & PstI
- Gunvant/Andy
    - Cloning wild type O3-33 using new PCT-F1 and PCT-R1 primers
    - Cutting at Xba & PstI
- **All assuming that the T7 promotor and rbs is contained between the suffix and prefix in the PSB1C3**
    - Sequence the region?

Conclusions: Waiting for primers, continue with cloning next week when primers arrive

August 1 Lab Journal

Scott:
Goals: Use new primers PCT-1F and 1R to create mutants with prefix and suffix to digest into plasmid. Need to pcr and digest.

PCR
-Used Q5, new primers, approx 1 ng gene block
-Anneal at 66C
-Gel Results:



Mutants aa88 and 173 worked. However yield was too low to digest, redone with oneTaq

Nithin/Yashes:
Goals: Finish Digestion,Ligation and Transformation
PC M 1,2,4,10,13

Digestion→ standard protocol. Xba/ Pst on gene. Spe/ Pst on plasmid.
Ligation → 1 hr at room temperature. 20 mins heat inactivation 65C.
Transformation → 1:1 all Mutants. Plated 200 uL.
Conclusion: Transformations didn't work.

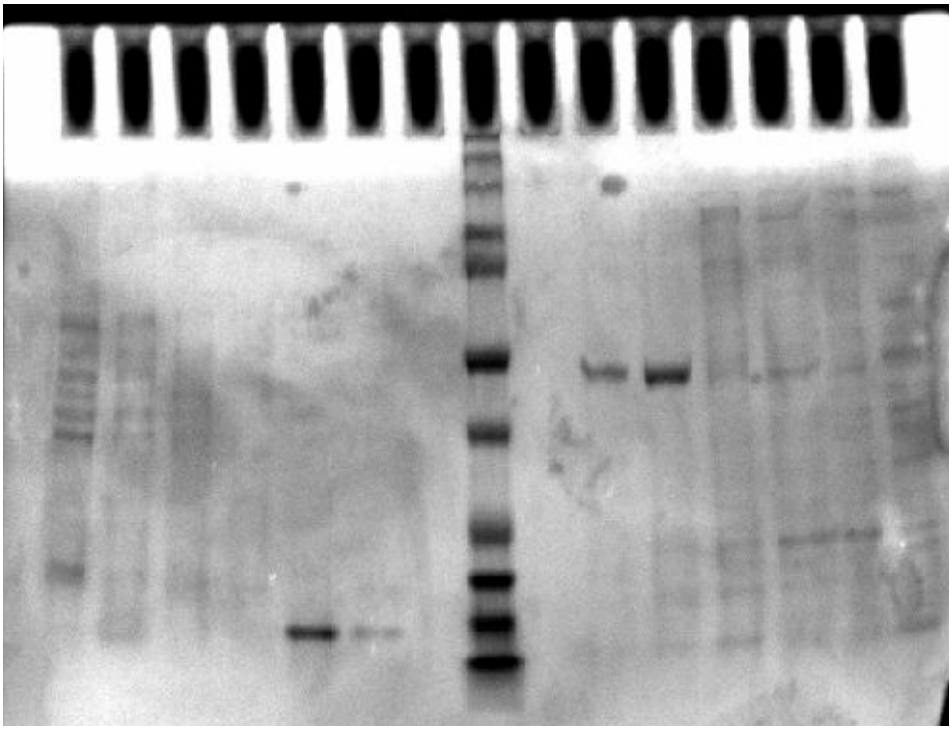
August 2 Lab Journal

Goals: Retransform cells and do a protein purification to test resin on PC 3.0 and O3-33

Protein Purification

Yashes:

- Purified O3-33 and Pcquad from pellet
    - Pellet size:
        - O3-33: .87 g
        - PCquad: 1.57 g
    - Cells lysed using lysis protocol outlined
        - 1:3 cell weight:lysis buffer used
    - Did not filter after pelleting cells after lysis
    - Proteins purified batch method
        - Washes: 25, 50, 150 mM imidazole
        - Elutions: 400, 400 mM imidazole
    - Ran SDS-PAGE:
        - Pcquad heated at 70 C for 10 minutes before loading, O3-33 was not heated
        - 15 uL of sample loaded in each well



- Resin regeneration using MES buffer worked

Transformation

Gunvant:

- Transformation worked. Ran colony PCR. All colonies showed the band. Innoculated 2 of the 5 colonies taken off plate.

Nithin:

- Retransformed all 5 mutants.

Conclusion: Regeneration worked so we can move on regenerating with this protocol. Transformation worked!

August 3 Lab Journal

Yashes:

- Ran native gel on O3-33 and Pcquad wild type

Nithin:

- Colonies Grew!!!
- Ran cpcr and it worked. Followed thermocycler protocol for cpcr. Extension time 1:15
- Inoculated 5mL culture for miniprep

Conclusion: We can with purifying mutants once sequences come in.

August 4 Lab Journal

Goals: Continue cloning

Yashes:

- Miniprepped and sent for sequencing:
    - 5 Pc Quad mutants: M1, M2, M4, M10, M13
    - O3-33 Wild Type
- Transformed Pcquad mutants and O3-33 wild type
- Ran colony pcr on O3-33 mutants aa88 and aa118
    - Failed: Plasmid at 500 bp → just psb1c3 without o3-33 gene
    - Need to start over :(
- O3 aa173 did not grow on plate: need to start over :(saayeeedd

Gunvant:

- Re ran PCR on O3-33 mutant aa88, aa113, aa173

Conclusion: Cloning continued.Waiting for sequencing results. Retransformed in case sequence was incorrect.

August 5

Yashes:

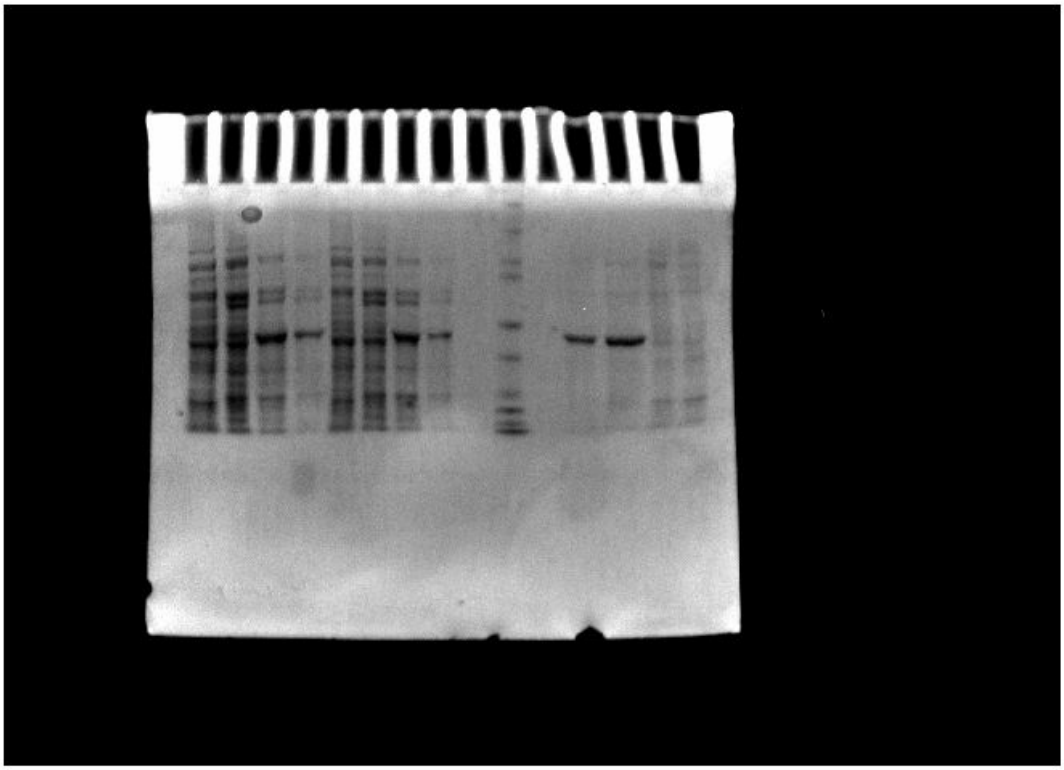
- Took out plates of PCquad mutants and O3-33 wild type
    - PCquad M1, M2, M4, and M13 grew
    - O3-33 wild type in psb1c3 grew
    - PCquad M10 did not grow → need to retransform

Conclusion: M13 and wildtype came in positive so get ready for expression. Others send in for more sequecing.

August 6 - 14 Lab Journal

Cell Growth

- Grew PC M 1 and PC M 13 following growing protocol
- Induced OD .8



PC Mutant 1 and Mutant 13 (not pure sample so regrow)

This week waiting on PC cloning and sequence verification of other mutants (M2, M4)
Also regrowing M10.
Conclusion: Purification was not pure so need to regrow M1 and M13 and also waiting for resequencing of M2,M4. M10 didnt purify at all.

August 15 Lab Journal

Goals: Sonicate cells grown from Friday and purify proteins

PC WT, M1, M2, M4, M10, M13 Purification:

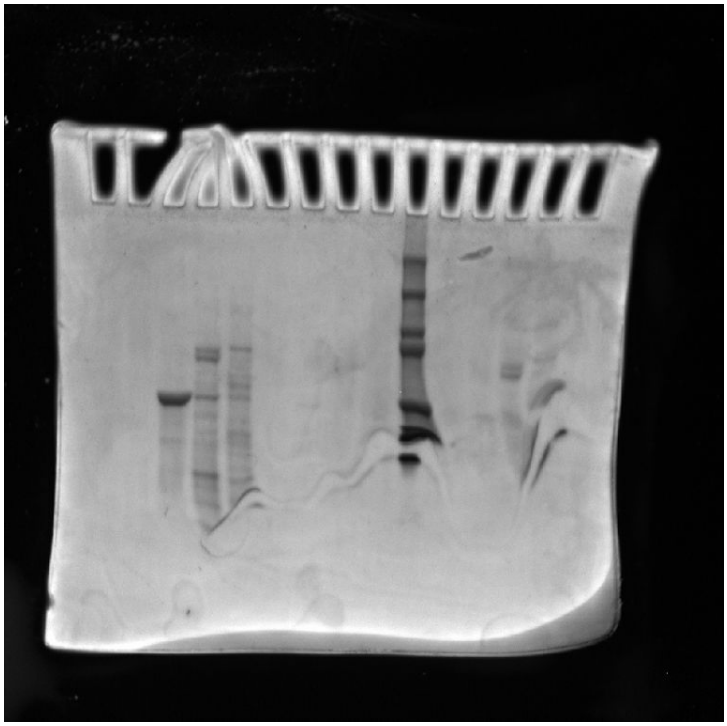
- Regenerated Ni resin with MES buffer (10 column volumes of MES, then 10 column volumes of pure water, stored in 1:1 20% ethanol)
- Sonication 10s, then rest 30s x10
- Column purification

    - 0.75mL column
    - EB: 20mM
    - Wash: 25mM, 50mM, 150mM
    - Elution: 400mM, 400mM (all 1.5mL)
- SDS Gel
    - 1:19 BME:Laemmli Sample for sample buffer
    - 10uL sample buffer + 10uL protein
    - Heat at 70C for 10min

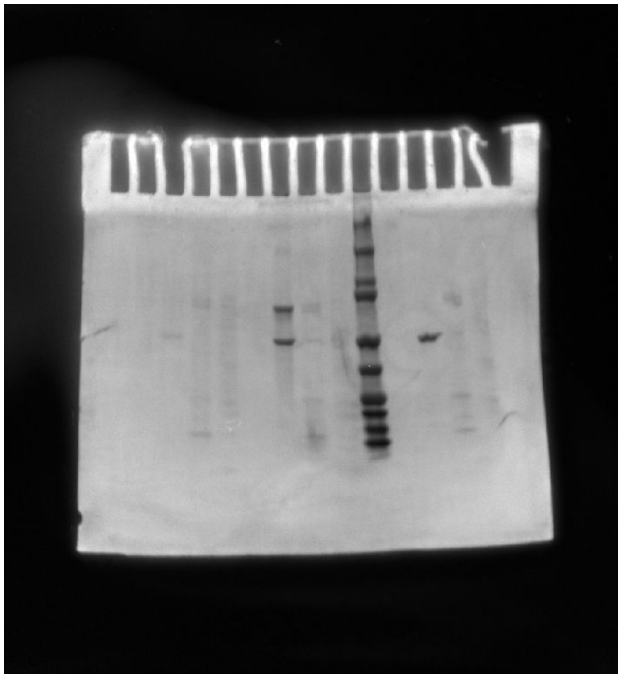
  Conclusion: Will run gel to verify purification.

August 16

Visualized PC WT and Mutants Gel



M13, M10, M4



M2, M1, PC

O3-33 Mutants sequence verified
Inoculated O3-33 and mutants

Conclusion: getting warped gel. Possibly because of buffer used. M2 didnt purify while M1 worked with some extra band. Possibly due to incomplete heating.

August 17

Grew O3-33 WT and Mutants aa88, aa118, aa173
Recharged and regenerated resin
Inoculated PC WT and Mutants M2, M4, M10
Conclusion: getting ready for another purification cycle.

August 18

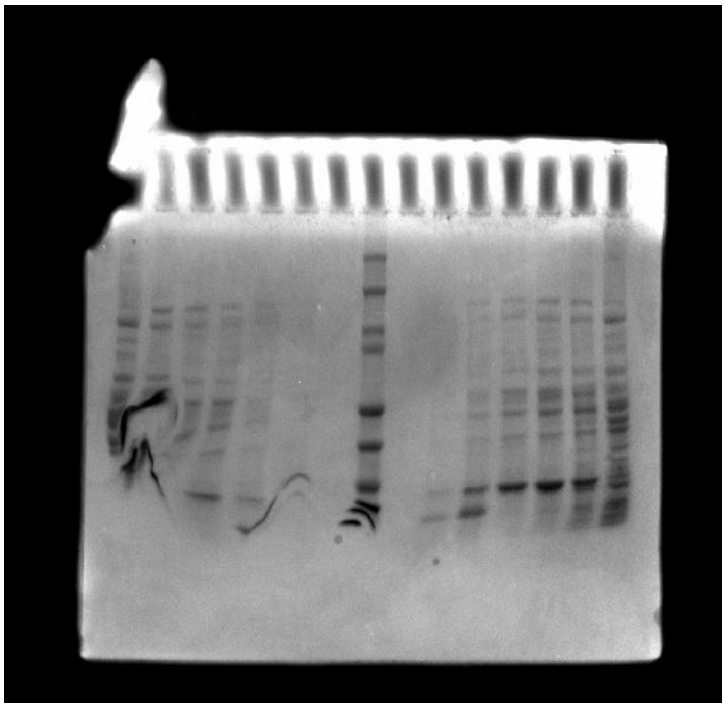
Lysed, purfied and ran SDS PAGE on O3-33 and mutants

- Batch method

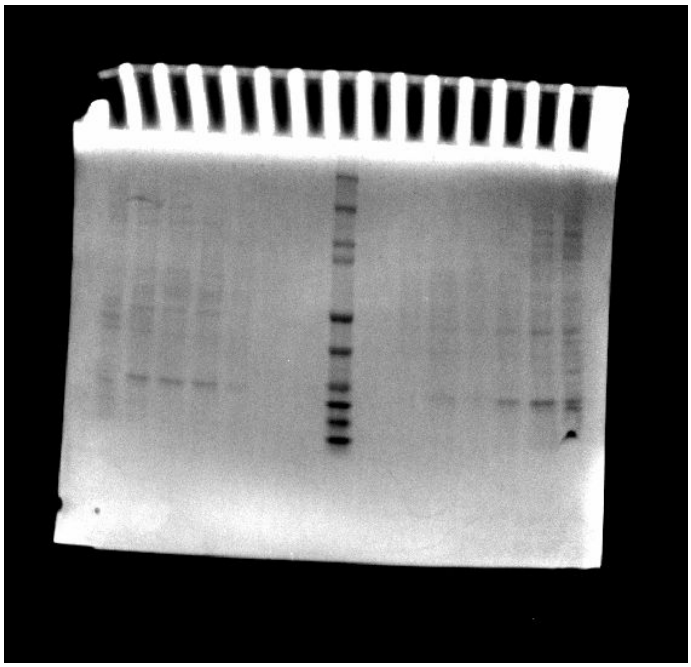
Grew PC WT, and mutants M2, M4, M10
Conclusion:Used batch method because ran out of columns. Buy more columns.

August 19

Visualized O3-33 WT, aa88, aa118 and aa173 Gel



O3-33, aa88

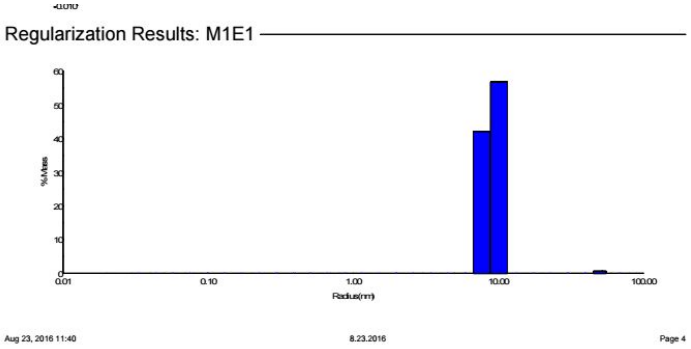


aa118, aa173

Conclusion: Fix Warp problem. CHANGE BUFFER. aa88 looks promising.

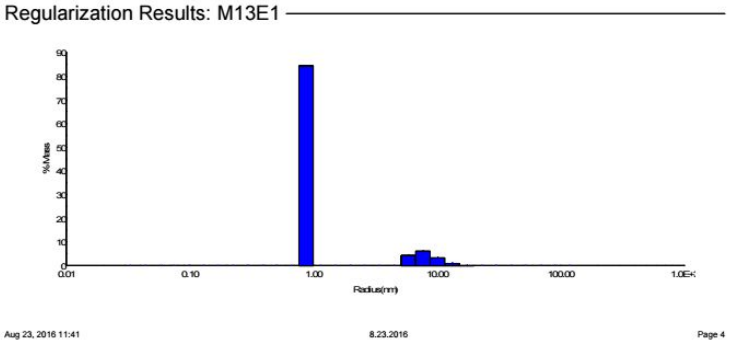
August 23rd

DLS M1 and M 13 cage



Regularization Results: M1E1 (continued)

	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	9.1	13.1	594	62.1	99.2
Peak 2	52.0	2.2	34820	37.9	0.8



Regularization Results: M13E1 (continued)

	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	0.9	0.0	2	0.4	84.5
Peak 2	8.2	27.9	465	65.9	15.4
Peak 3	63.6	27.5	55759	33.8	0.1

Conclusion:M1 FORMED!!!!! M13 did not… from DLS results.

August 25th

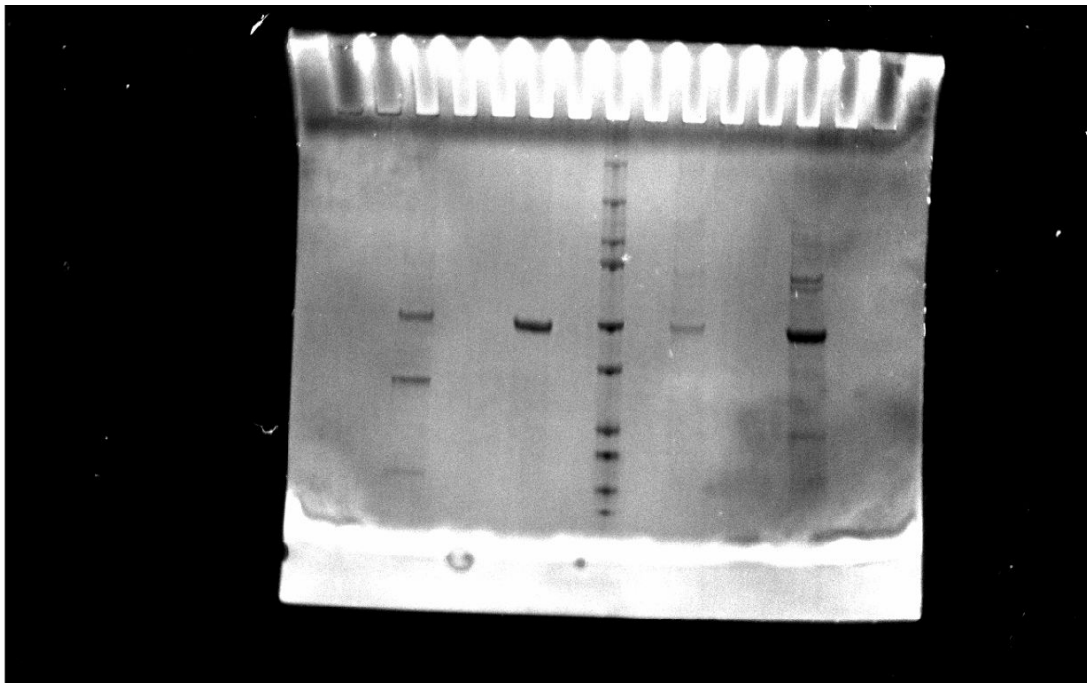
Ran thrombin Assay on M1 and Used wildtype PC as control

- Used .2mg of M1 and .2 mg of Wildtype
- 1.5 units of Thrombin
- Total volume of reaction was 200 uL
- 22C 16 hour incubation

Conclusion: Thrombin Assay based on Thrombin paper.

August 26

- Ran gel on PC and M1 from the thrombin assay

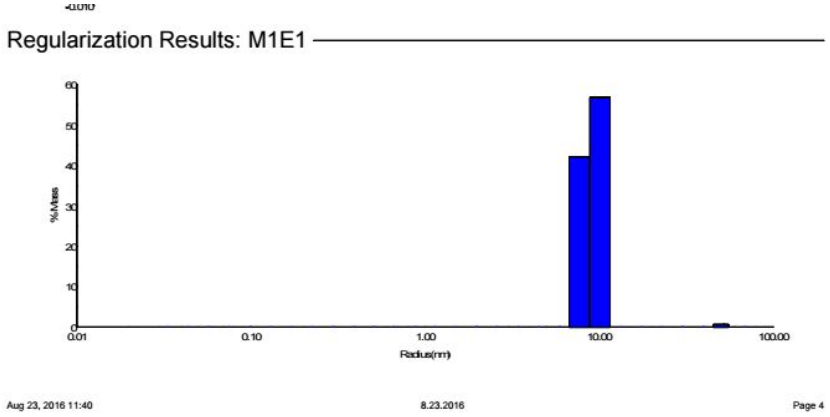


M1 with thrombin, M1 without thrombin, ladder, PC with thrombin, PC without thrombin

Conclusion:OMG!!! THROMBIN CUT INTO THE TWO PIECES WE WANTED IT TO CUT!!!

August 29

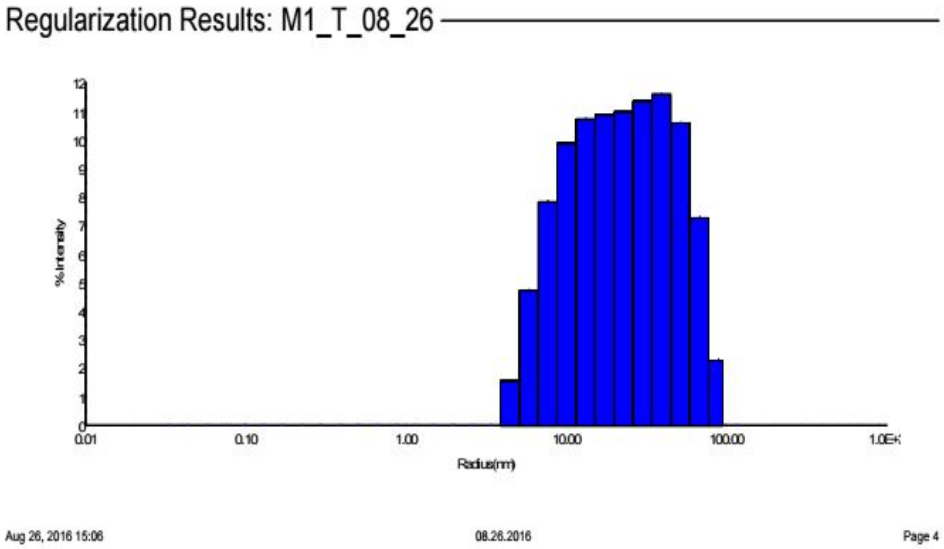
- Ran DLS on Thrombin assay M1 and PC



Regularization Results: M1E1 (continued)

	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	9.1	13.1	594	62.1	99.2
Peak 2	52.0	2.2	34820	37.9	0.8

M1 Without Thrombin



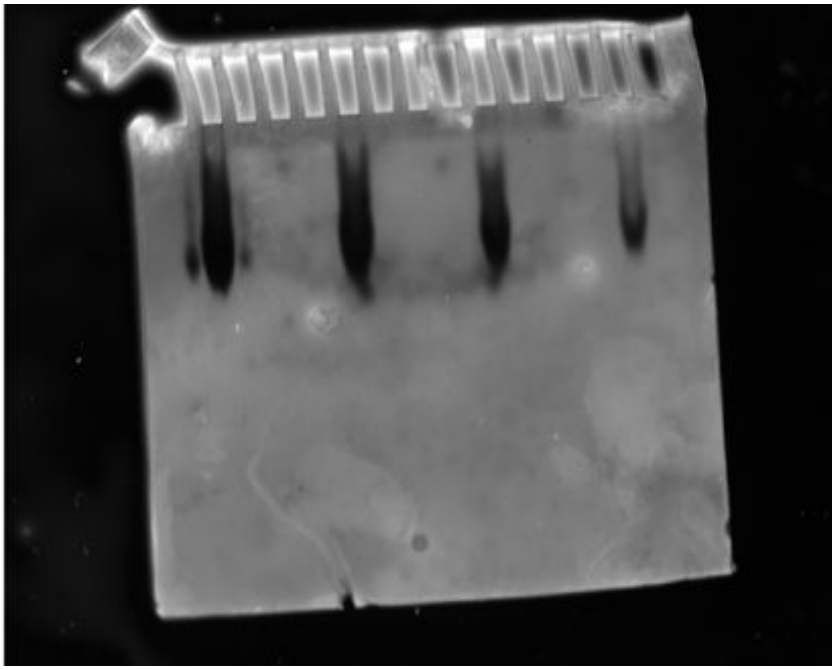
Regularization Results: M1_T_08_26 (continued) —

	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	28.4	71.8	8465	100.0	100.0

M1 With Thrombin
Conclusion: DLS confirms results that wildtype did not disassemble in presence of thrombin while mutant protein did

August 30

- Ran Native gel control to test Native Gel conditions with Albumin



20 mg/ml, 10 mg/ml, 5 mg/ml, 1mg/ml

September 2-3

- Inoculated and grew O3-33WT, and O3-33 mutants 88, 118, and 173 and PCQuad WT and PC M1 because previously purified samples were not viable. Also need to test mutant O3-33 cages
- Conditions:
  Growth and Pelleting:

1. Inoculate 5 ml of cells in 37 C overnight
2. Incubate cells in larger flask(s) (1 - 4 L per batch) in 37 C until OD reaches 0.8
3. Add 0.5 mM IPTG (1 mL of 0.5 M stock per liter of cells)
4. Incubate cells in 30 C for 5 hrs
5. Aliquot cells into 50 mL Falcon tubes
6. Wash and pellet cells
    1. Centrifuge cells 4000g for 10 min
    2. Dump out supernatant
    3. Resuspend cells in 10 ml of cold water
    4. Consolidate suspended pellets into fewer falcon tubes
    5. Centrifuge… Repeat until all cells washed into a single tube

Lysis and filtration:

1. Resuspend cell pellet in lysis buffer (1 : 2 cell weight : ml of lysis buffer)
    1. PCQuad: 20mM sodium phosphate (ph=8.0), 300 mM sodium chloride, 10 mM imidazole
    2. O3-33: 50 mM TRIS (ph=8.0), 250 mM NaCl, 20mM imidazole
2. Chill cell suspension for 10 min
3. Add 10 uL 100 mM PMSF for every mL of cell suspension right before sonication
4. Sonicate cell suspension:for 10 sec (10 cycles; 30 sec pause between each cycle)
5. Centrifuge lysates for 30 min at 13000 rpm
6. Filter supernatant through a 22 um Millipore filter

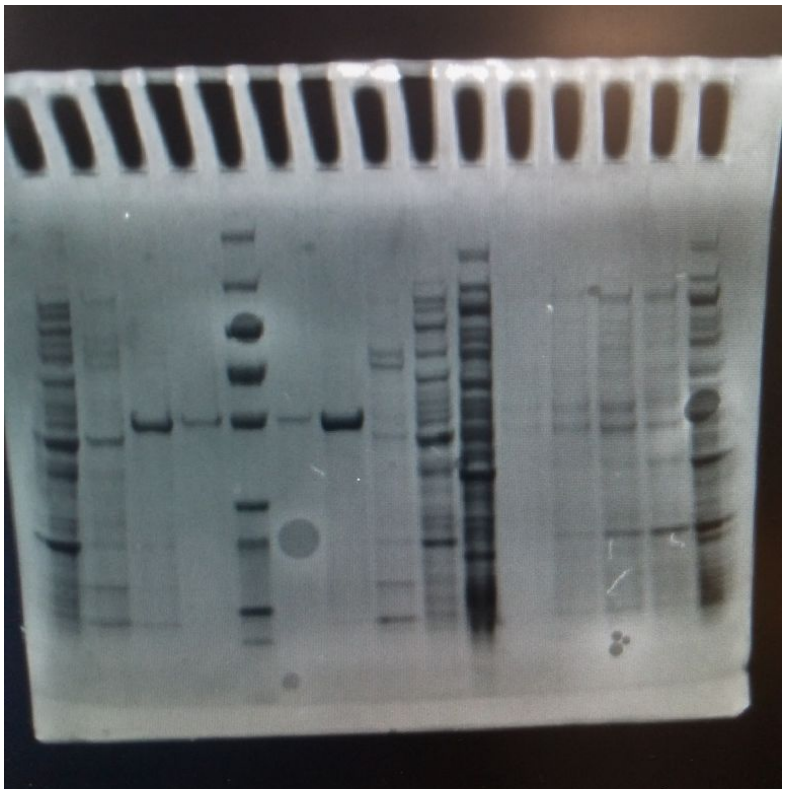
September 4

- Purified O3-33 and mutants and PCquad and PC M1
- Conditions:
- Used Hispur column purification protocol
    - Add appropriate amount of resin bed to purification column
    - Equilibriate column with two resin bed volumes of equilibration buffer
    - Add equal volume of equilibration buffer through filtered protein sample

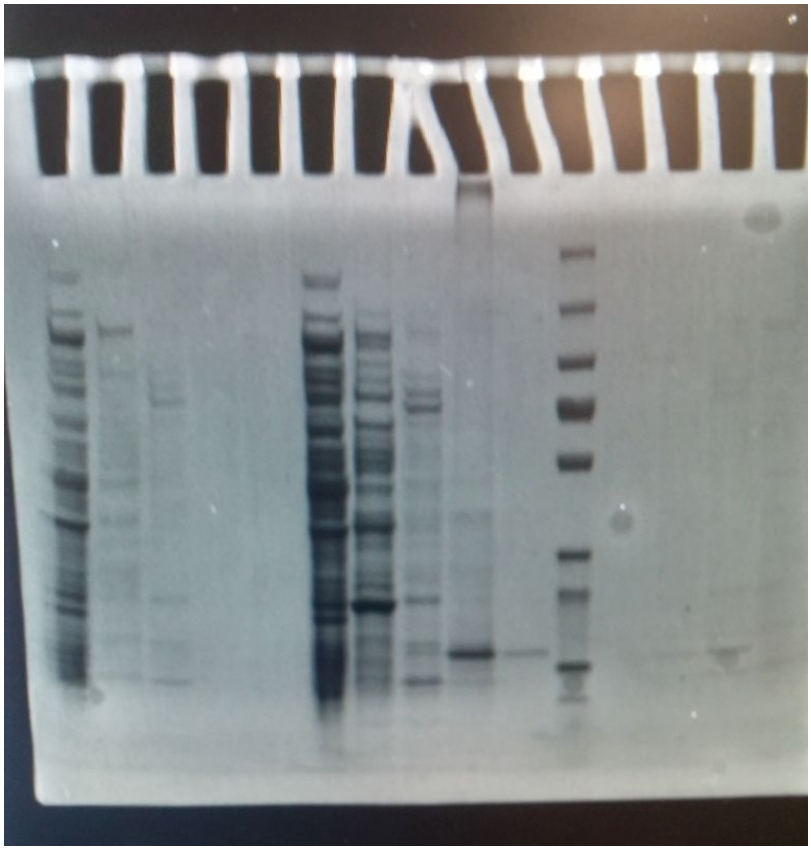
- Add sample through column. Collect flow through and reapply through column. Save flow through for downstream analysis if desired
- Wash resin with two resin bed volumes of wash buffer with a gradient of imidazole concentrations until protein A280 reaches baseline Save flow through for downstream analysis if desired
- Elute protein with two resin bed volumes of elution buffer. Repeat elution. Save flow through for downstream analysis if desired
- Imidazole concentrations used:
    - PCQuad
        - Equilibration: 20 mM
        - W1: 25 mM, W2:40 mM, W3: 150 mM
        - E1: 400 mM, E2: 400 mM
    - O3-33
        - Equilibration: 20 mM
        - W1: 30 mM, W2: 40 mM, W3: 50 mM
        - E1: 400 mM, E2: 400 mM

September 5

- Ran gel on purified O3-33 and mutants and PCQuad and M1



PC, ladder, M1, O3 173

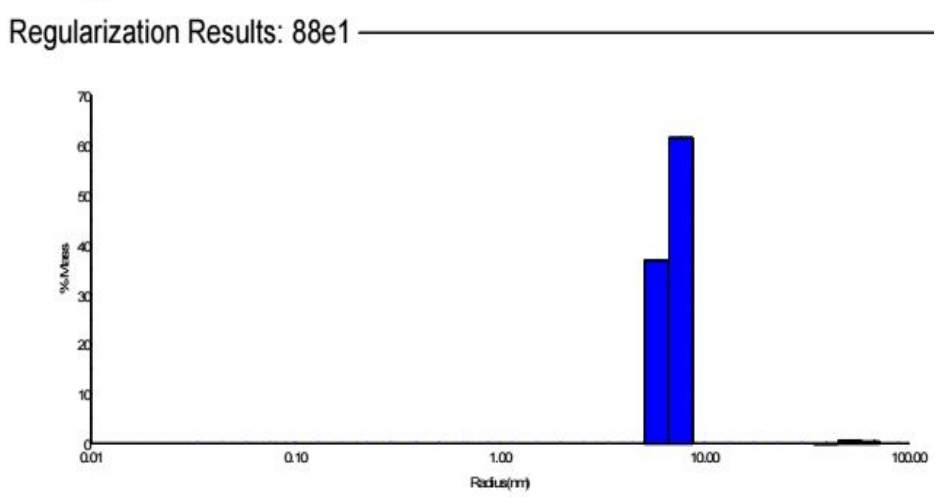


O3 118, O3 88, ladder, O3 WT

Conclusion: aa88 continues to look promising. Need to send out to DLS.

September 7

- Ran DLS on O3-33 aa88



	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	7.0	12.7	322	32.0	98.7
Peak 2	58.4	14.8	45780	68.0	1.3

Conclusion:O3-33 aa88 cage formed!

September 10-11

Ran an overnight thrombin assay on PC WT, PC M1, O3-33 WT, O3-33 aa88

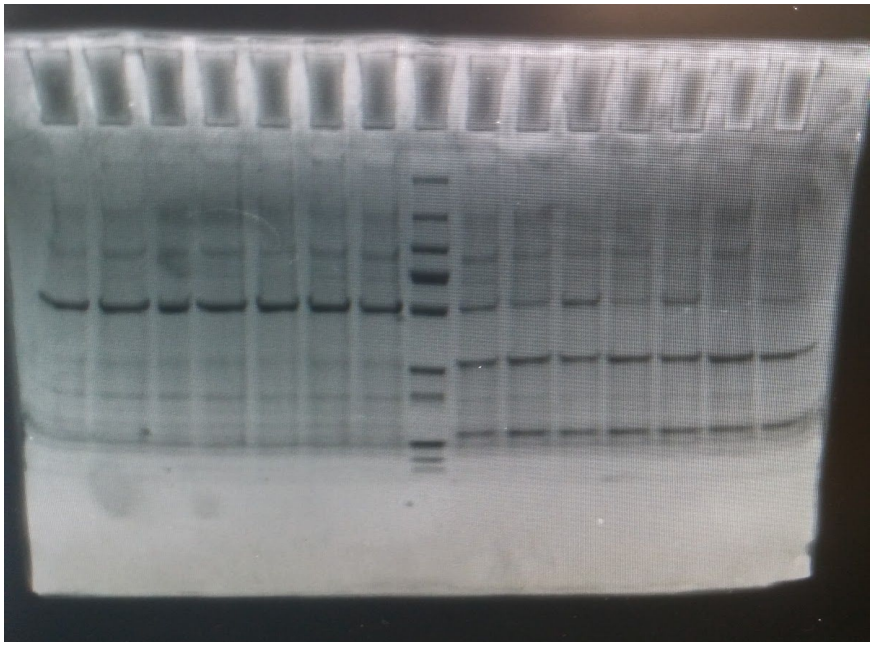
First Run: 4 samples, 2 of pc and m1 each. Thrombin was added to 1 of pc and m1 samples.

Protein was at concentration of .1 mg/mL. We had 200 uL so 20ug protein. We added 1.5 uL of thrombin at 460 units/mL so we added .69 units, about 3.5 times the needed amount.

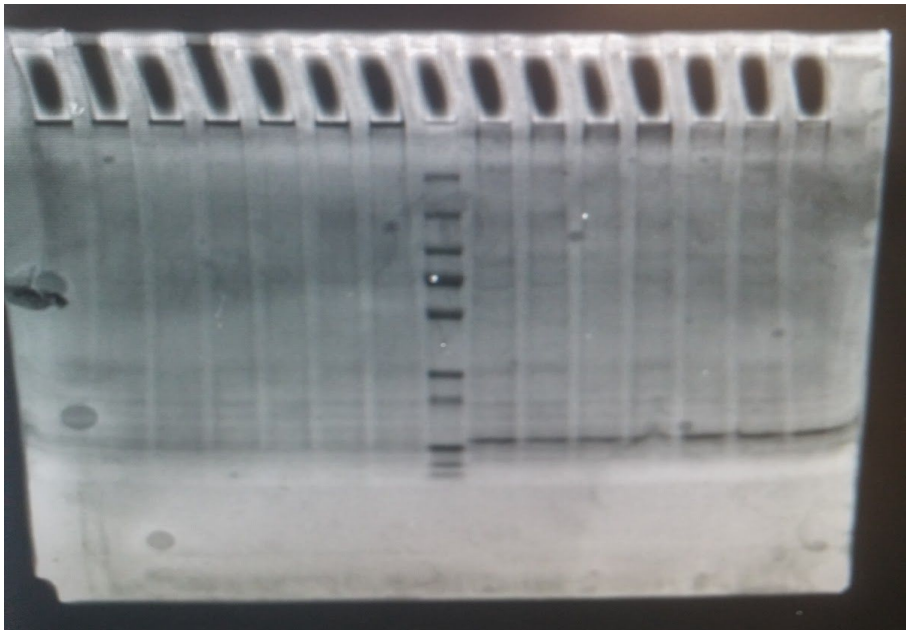
Second Run: All protein samples at concentration of .2mg/mL. 36 samples run. 9 per for m1, pc, o3, and m88. For each cage, one no thrombin sample was run, as were 2 every 4 hours at 4, 8, 12, and 16 hours with thrombin. Of these 2 samples per time point, the units added were 3 and 5 times as much thrombin needed into 60uL of sample.

September 12

- Ran gel on all samples of thrombin assay



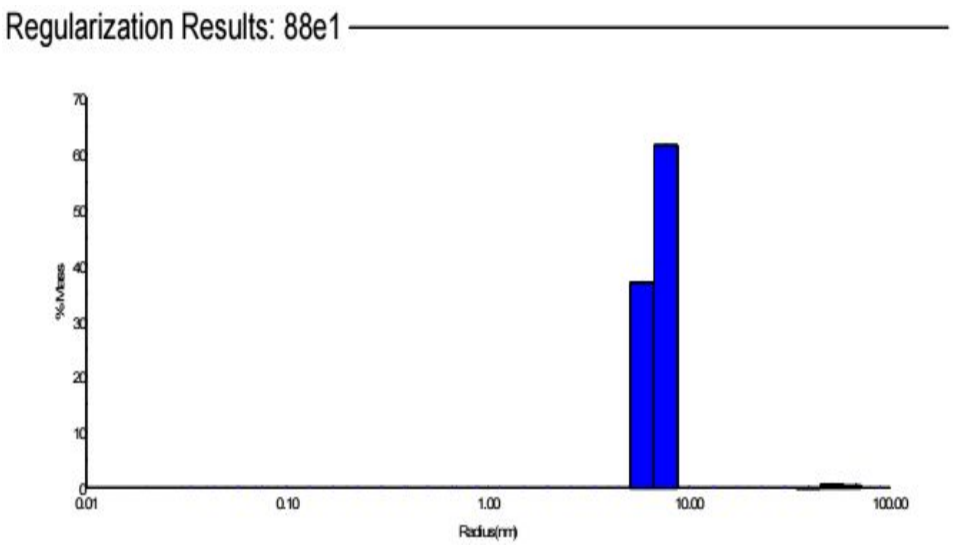
PC, M1 -- longer time in thrombin on outside of gel



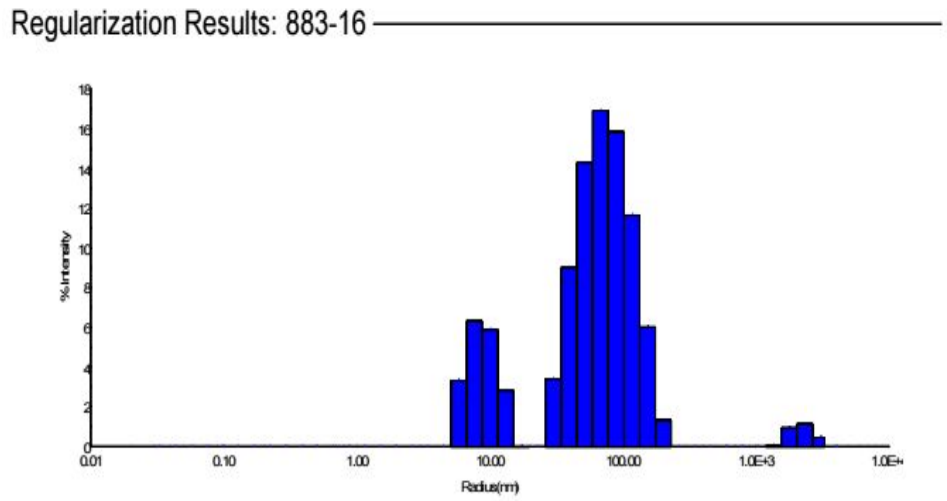
O3-33 aa88, O3-33 WT -- longer time in thrombin on outside of gel
We see aggregation of cut aa88 at top
Conclusion: Thrombin cleavage worked exactly the way we expected, where the longer the cutting along with the most concentration cut the most.

September 13

- Ran DLS on all samples in thrombin assay



O3-33 aa88 without thrombin



O3-33 aa88 with thrombin (cut cage)

<u>Conclusion: DLS results confirm cage disassembly of 03-33.</u>